FULL PAPER *Wildlife Science*

Annual ovarian activity monitored by the noninvasive measurement of fecal concentrations of progesterone and 17β-estradiol metabolites in rusa deer (*Rusa timorensis*)

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ABSTRACT. To clarify the reproductive cycle of female Rusa deer (*Rusa timorensis*), the fecal concentrations of progesterone and 17β-estradiol metabolites were measured. Fecal samples were collected on a weekly basis for one year (between October, 2012 and September, 2013) from five healthy adult hinds in Thailand. At the beginning of the study, three hinds were pregnant. Two hinds delivered one healthy offspring, and one hind delivered a stillborn calf. The mating period of Rusa hinds in Thailand is from November to April. In pregnant hinds, fecal progesterone metabolite concentration was high in late pregnancy and abruptly declined to the baseline around parturition, suggesting that the placenta secretes a large amount of progesterone. Fecal 17β-estradiol metabolite concentration remained elevated around the day of parturition. Both concentrations of fecal progesterone and 17β-estradiol metabolites in non-lactating hinds were significantly higher than those in lactating hinds, indicating that ovarian activity of lactating hinds is suppressed by the suckling stimulus of fawn during lactation. The present study demonstrated that monitoring of fecal steroid hormones is useful method for assessing ovarian function in this species.
KEY WORDS: fecal estradiol metabolite, fecal progesterone metabolite, pregnancy and lactation, reproductive cycle, Rusa deer

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Rusa deer (*Rusa timorensis*) are an endemic to the Indonesian Archipelago. According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, Rusa deer are considered as vulnerable due to their highly fragmented natural habitats, habitat degradation and the effects of poaching [11]. However, the species has been introduced to many countries in Indo-Pacific region including Thailand for venison and to be farmed for velvet production [24]. It is therefore one of six species in the Cervidae family living in Thailand. Thus, the understanding of Rusa deer reproduction not only has economic benefits for deer farming operations, but also represents a model for endangered deer species in Thailand. The major breeding activity was observed in plasma progesterone of female Rusa deer in Malaysia under appropriate environmental conditions and high nutrition in March to July [14]. After 8 months of gestation, adult hinds generally give birth with a single calf. In general, the body weight of Rusa hind at the first mating (18–20 months of age) is approximately 46 kg [30] and increases to 60 kg when mature. Mature stag weighes approximately 80 kg [9]. However, there is little reproductive information or endocrine data to confirm seasonal ovarian activity in Rusa hind, and there is no information on fecal steroid hormone profiles of Rusa deer. The objective of this study was to determine the reproductive cycle of Rusa hind by monitoring the annual fecal concentrations of progesterone and 17β-estradiol metabolites.

MATERIALS AND METHODS

Animals and management: Five healthy adult Rusa hinds (Fig.1), aged 2–3 years old with live body weight of 32–45 kg, were maintained at Kasetsart University Kamphaeng Campus, Thailand (14° 0′ 24.60″ N 99° 59′ 19.80″ E). Hinds were separated into two groups; pregnant (n=3) and non-pregnant (n=2). All animal procedures were approved by the Deer Co-operative of Thailand, LTD. (DCOT). All hinds were exposed to natural photoperiod and fed *ad libitum* with

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Fig. 1. A healthy adult Rusa hind (*Rusa timorensis*) in August.

fresh Panicum grass (*Brachiaria mutica*), Pangola grass (*Digitaria eriantha*), commercial pellets [Betagro 004 dairy cattle pellet (16% protein, 3% fat, 12% fiber and 13% moisture); Betagro Co. Limited, Bangkok, Thailand] and natural leaves. They were allowed to assess tap water *ad libitum*.

Sampling period and fecal collection: Fecal samples were collected weekly from all animals all year round from October 2012 through to September 2013. Five hinds were housed together in outdoor enclosures adjacent to stags. Fresh feces were collected between 08.00 to 09.30 hr and kept in sealed plastic bags. All fecal samples were stored at −20°C until processing.

Pregnancy and fawning data: Three of five hinds were pregnant in the last season. Two hinds delivered one healthy offspring on October 15 and October 20, 2012, respectively. Two fawns were housed with their mother until weaning. Fawns were weaned until 6 months old. Another hind delivered a stillborn calf on October 18, 2012. However, this hind was lactated by two fawns delivered from two different mothers. This species of deer nursed not only their own fawns but also other fawns delivered from females in the same group.

Fecal extraction: Frozen fecal samples were dried in an oven at 56–60°C for approximately 72 hr and were extracted with ethanol following procedure that described by Brown *et al.* [3]. Sample contained with steroid metabolites was stored at −20°C until analysis.

Fecal progesterone analysis by enzyme immunoassay (EIA): Fecal concentration of progesterone metabolite was determined by enzyme immunoassay as described by Brown *et al.* [5]. The intra- and inter-assay coefficients of variation (CV) were 6.27% and 6.28% (n=11), respectively. Data are expressed as *n*g/g dry feces.

Fecal 17β-estradiol analysis by radioimmunoassay (RIA): The concentration of fecal 17β-estradiol metabolite was determined by radioimmunoassay using ^{125}I-labeled radioligand as described by Taya *et al.* [29]. Anti-sera against 17β-estradiol (GDN 244) were used. The intra- and inter-assay coefficients of variation (CV) were 4.64% and 7.60% (n=10).

Data evaluation and statistical analyses: Results of five hinds were analyzed by their pregnancy status. The female Rusa deer do not show a clear seasonality in reproductive behavior. On the other hand, the antlers of male Rusa deer shedded in May, and the new velvet antlers grew from June to October. Thereafter, the antlers became hard from November to April [18]. Mating behavior was observed from November to April. No males are available to mate females when male Rusa deer have velvet antlers. Males are presumed to be sexually active and fertile in the hard antlers stage [18]. From these observations in Kasetsart University, Kamphaeng Saen Campus, Thailand, the breeding season and non-breeding season were determined during November to April and May to October, respectively, in the present study.

For each female, baseline progesterone and 17β-estradiol metabolites values were calculated using an iterative process in which values that exceeded the mean plus 1.5 standard deviations (SD) were excluded. This process was recalculated until no values could be removed. Baseline values were those remaining after exclusion of all high values [4, 22]. Fecal concentrations of progesterone and 17β-estradiol metabolites during pregnancy were not included in calculation of baseline value. The difference in fecal concentrations of progesterone and 17β-estradiol metabolites in the breeding season (November-April) and the non-breeding season (May-October) was compared using a two sample Student *t*-test. The ovarian activity was defined as the fecal concentrations of progesterone and 17β-estradiol metabolites. If the values of progesterone metabolite were greater than the baseline, they were considered as a luteal phase, while the values were less than the baseline progesterone values, they were considered as an inter-luteal phase. [13]. Fecal concentrations of progesterone and 17β-estradiol metabolites were compared by using the two-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests. The data were presented as mean ± standard errors for mean (SEM). An analysis of hormone concentrations was performed using SPSS (SPASS Inc., Chicago, IL, U.S.A.). Values of $P<0.05$ were considered statistically significant.

RESULTS

Characterization of progesterone and 17β-estradiol metabolites assays: Displacement curves obtained for fecal samples of female Rusa deer in the progesterone metabolites EIA and 17β-estradiol metabolites RIA are shown in Fig. 2. In the progesterone EIA, addition of fecal sample of two female Rusa deer resulted in displacement of progesterone tracer in a dose-response manner. Dose-dependent curves of serially diluted fecal samples showed parallelism to the standard curves of progesterone (Fig. 2a). In the 17β-estradiol metabolites RIA addition of fecal samples of two female Rusa deer also resulted in displacement of 17β-estradiol tracer. There was a good dose-response relationship between the standard curve of 17β-estradiol and fecal samples (Fig. 2b).

Fecal concentrations of progesterone and 17β-estradiol

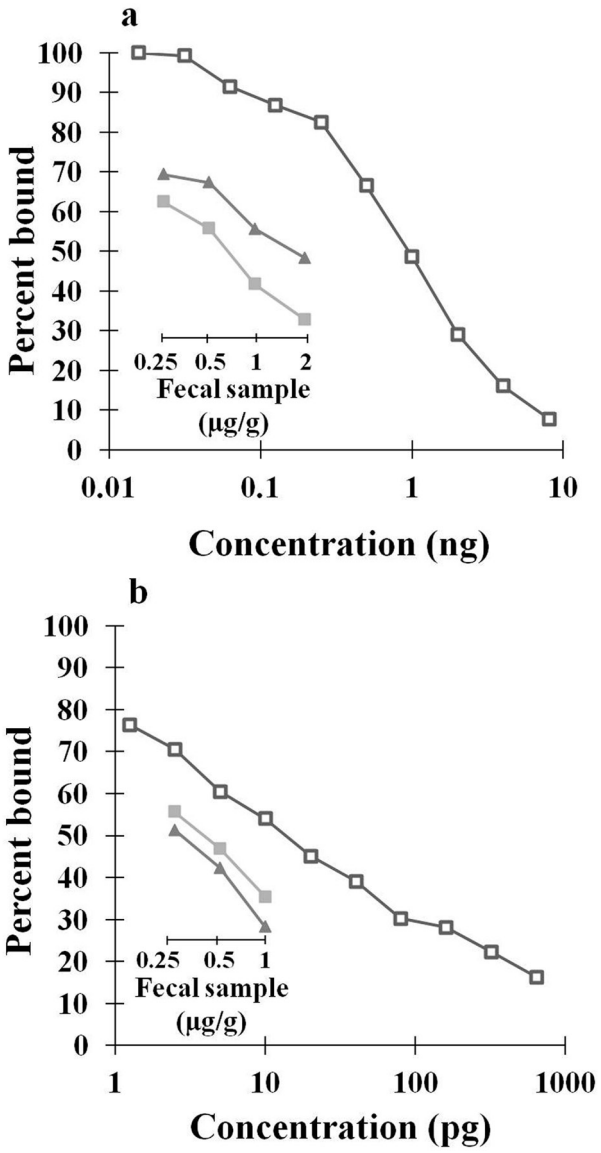

Fig. 2. Dose response curves of fecal samples of Rusa deer in progesterone enzyme immunoassay (a) and in 17β-estradiol radioimmunoassay (b). Each value represents the mean of duplicate determinations in Rusa deer fecal samples (▲, □) and triplicate determinations in standard (□).

metabolites during the late stage of pregnancy and postpartum: At the beginning of the study, three hinds were pregnant. Mean fecal concentrations of progesterone and 17β-estradiol metabolites of three animals during the late stage of pregnancy period and the postpartum period after delivery are shown in Fig.3. The highest progesterone concentration was shown in the late stage of pregnancy, followed by a sharp decline around delivery (data not shown). The concentrations of progesterone metabolite during the late stage of pregnancy were 37.4–21.54 times greater than baseline and significantly higher than that of the postpartum period. On the other hand, the fecal concentration of 17β-estradiol metabolite increased during the late stage of pregnancy and peaked on the day of parturition and followed by a decline after delivery (data not shown). Peak concentrations of fecal 17β-estradiol metabolite on the late pregnancy were 4.56–5.97 times greater than baseline and significantly higher than that of the postpartum period.

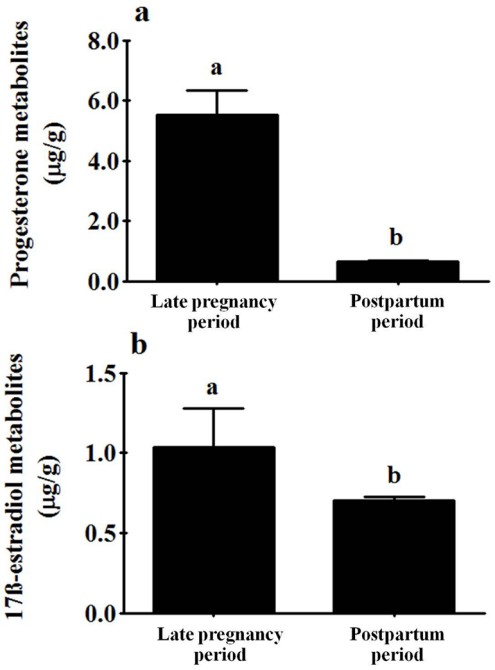

Fig. 3. Mean (± SEM) of fecal concentrations of progesterone and 17β-estradiol metabolites during the late stage of pregnancy (n=13) and postpartum (n=143). Different letters indicate significant differences among period (P<0.05) by Duncan's multiple comparison tests.

Fecal concentrations of progesterone and 17β-estradiol metabolites in the lactating and non-lactating animals: Fecal concentrations of progesterone and 17β-estradiol metabolites in lactating and non-lactating hinds are shown in Fig. 4. Fecal concentrations of progesterone and 17β-estradiol metabolites in non-lactating groups were significantly higher than lactating groups (P<0.05). Fecal concentrations of progesterone and 17β-estradiol metabolites of lactating and non-lactating hinds in the breeding season and the non-breeding season are shown in Fig. 5. The fecal concentration of progesterone metabolite of the lactating hinds in non-breeding season was significantly higher than that in breeding season. It is also true in the non-lactating groups (Fig. 5a). In contrast, on fecal concentration of 17β-estradiol metabolite in non-lactating groups, the level in the breeding season was significantly higher than that in non-breeding season (Fig. 5b). In the lactating groups, the fecal concentration of 17β-estradiol metabolite tends to be higher in the breeding season than non-breeding season, but there was no significant difference between two groups (Fig. 5b).

Correlation plots of fecal concentrations of progesterone

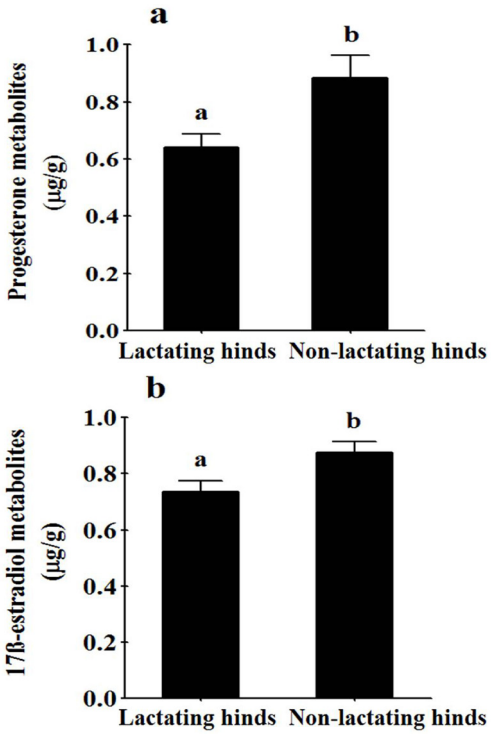

Fig. 4. Mean (± SEM) of fecal concentrations of progesterone (a) and 17β-estradiol (b) metabolites between lactating (n=143) and non-lactating (n=104) Rusa hinds. Different letters indicate significant differences between each hind (P<0.05) by Duncan's multiple comparison tests.

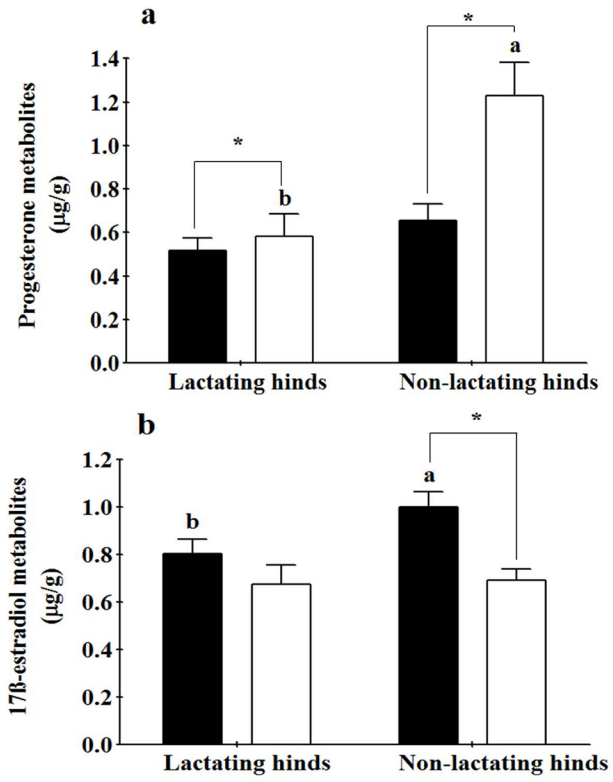

Fig. 5. Mean (± SEM) of fecal concentrations of progesterone (a) and 17β-estradiol (b) metabolites in lactating (■; n=77, □; n=60) and non-lactating (■; n=66, □; n=44) Rusa hinds. The black and white bars indicate the breeding season and non-breeding season, respectively. The data during pregnant stages were not included. Asterisks represent significant difference between the breeding and non-breeding seasons (P<0.05), and different letters represent significant differences within lactating and non-lactating hinds (P<0.05) by Duncan's multiple comparison tests.

and 17β-estradiol metabolites in all Rusa hinds are shown in Fig. 6. The fecal concentrations of progesterone and 17β-estradiol metabolites were significant negative correlation. The Spearman's correlation coefficient was r=−0.12, n=247 (Fig. 6).

DISCUSSION

Reproductive information is essential for the development of management strategies of species in captivity. It can be adapted in conservation programs to other endangered species. In the present study, the reproductive cycle of Rusa deer in Thailand was investigated by monitoring annual fecal concentrations of progesterone and 17β-estradiol metabolites. Three of five female Rusa deer were pregnant at the beginning of the present study. Therefore, fecal concentrations of progesterone and 17β-estradiol metabolites were monitored during the late pregnant, lactating and non-lactating periods. These results demonstrated that concentrations of fecal progesterone metabolite were high in the late pregnancy and abruptly declined to the baseline level around parturition. Peak levels of fecal concentration of progesterone in the late pregnancy were averaging about more than 20 times high as compared with the level in the lactating period. These results strongly suggested that the placenta of Rusa deer secretes a large amount of progesterone. On the other hand, fecal concentration of 17β-estradiol began to increase in the late pregnancy, remained elevated at the day of parturition and then decreased to the baseline level in the lactating period. In mammals, estrogens were increase during pregnancy, especially in late pregnancy, such as domestic ungulates (mares [15] and goats [12]), laboratory rodents (rats [25] and golden hamster [17]) or wild mammals (Japanese monkeys [16, 23], red deer [2], white-tail deer [10] and reindeer [20]). High levels of circulating estrogens promote production of oxytocin receptor [34] and relaxin receptor [6] to assist preparation of the reproductive tract for parturition and subsequently lactation. These results suggest that early follicular development occurs during the postpartum period in this deer, such as mares [15] and rats [25]. A future study will examine ovarian follicular development using ultrasonography to determine if a similar postpartum ovarian activity exists in Rusa deer.

In the previous study, the estrous cycle length during the breeding season of the female Rusa deer, inhabiting tropical Peninsular Malaysia, was 19.2 days, based on plasma concentration of progesterone [14]. A future study will examine detail pattern of fecal progesterone and 17β-estradiol me-

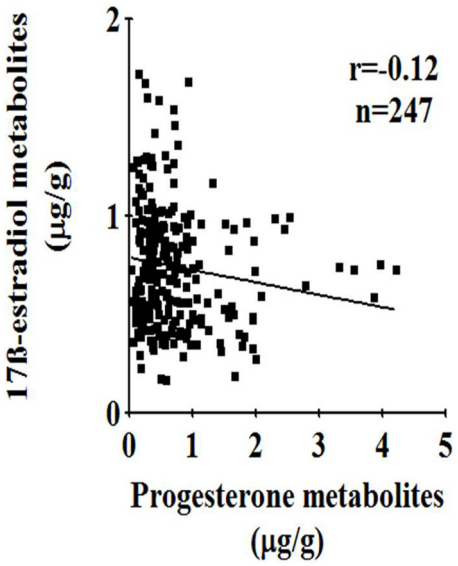

Fig. 6. Correlation plots of fecal concentrations of progesterone and 17β-estradiol metabolites in Rusa hind. The data during pregnant stages were not included.

tabolites by more frequent sampling to determine exact the estrous cycle length of Rusa deer in Thailand. The previous study demonstrated that male Rusa deer have a clear seasonality of reproductive function with their antler growth cycle [18]. Male Rusa deer showed most mating activity between November and April concomitant with timing of hard antler periods. In the hard antler periods, circulating testosterone increased, and males became aggressive [18]. These results demonstrate that the mating period of Rusa hind in Thailand is from November to April. In the present study, therefore, the breeding season was determined during November and April, and the non-breeding season was during May and October. Similar characteristic in reproduction of Rusa deer was also confirmed in Malaysia [Personal communication with Prof. Abd Wahid Haron, Jabatan Pengajian Klinikal Veterinar, Fakulti Perubatan Veterinar, Universiti Putra Malaysia].

In the present study, during the postpartum period, fecal concentrations of progesterone and 17β-estradiol metabolites in lactating hinds were lower than non-lactating hinds. These results indicated that ovarian activity of lactating hinds is suppressed by the suckling stimulus of fawn during lactation. The secretion of progesterone and 17β-estradiol is depressed by the suckling stimuli of a fawn through the suppression of secretion of kisspeptin and gonadotropin-releasing hormone from the hypothalamus, and the secretion of gonadotropins from pituitary gland [26, 28, 32, 33]. The suckling stimulus by the fawn also promotes the hypothalamus-pituitary-adrenal axis [1, 26, 27]. Different hormone concentrations in individual hind are also affected by nutrients and body weight [7, 8, 19, 21, 31]. The present study suggested that Rusa hinds have the ability to breed the early stages of lactation as well as rats [25] and mares [17]. More studies are required to clarify mechanism responsible for postpartum ovarian function during lactation in this species.

In summary, the present study suggests that female Rusa deer is not seasonal breeder whereas male Rusa deer is strongly seasonal in Thailand. The present findings suggest that female Rusa deer may be possible to utilize assisted reproductive techniques procedures throughout the year. With this information, future studies will focus on developing techniques, such as artificial insemination and embryo transfer, to create genetically healthy population and produce offspring for future reintroduction of endangered deer. In addition, non-invasive technique of measuring fecal steroid hormones is a useful method for evaluating reproductive endocrinology in Rusa deer.

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